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Molecular Pathology of Fatal Familial Insomnia

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Fatal familial insomnia (FFI) is linked to a mutation at codon 178 of the prion protein gene, coupled with the methionine codon at position 129, the site of a methionine/valine polymorphism. The D178N mutation coupled with the 129 valine codon is linked to a subtype of Creutzfeldt-Jakob disease (CJD^{178}) with a different phenotype. Two protease resistant fragments of the pathogenic PrP (PrP^{res}), which differ in molecular mass, are associated with FFI and CJD^{178}, respectively, suggesting that the two PrP^{res} have different conformations and hence they produce different disease phenotypes. FFI transmission experiments, which show that the endogenous PrP^{res} recovered in affected syngenic mice specifically replicates the molecular mass of the FFI PrP^{res} inoculated and is associated with a phenotype distinct from that of the CJD^{178} inoculated mice, support this idea. The second distinctive feature of the FFI PrP^{res} is the underrepresentation of the unglycosylated PrP^{res} form. Cell models indicate that the underrepresentation of this PrP^{res} form results from the PrP dysmetabolism caused by the D178N mutation and not from the preferential conversion of the glycosylated forms. Codon 129 on the normal allele further modifies the FFI phenotype determining patient subpopulations of 129 homozygotes and heterozygotes: disease duration is generally shorter, insomnia more severe and histopathology more restricted to the thalamus in the homozygotes than in the heterozygotes. The allelic origin of PrP^{res} fails to explain this finding since in both cases FFI PrP^{res} is expressed only by the mutant allele. Despite remarkable advances, many issues remain unsolved precluding full understanding of the FFI pathogenesis.

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Introduction

In addition to the study of the clinical features and the sleep mechanisms associated with fatal familial insomnia (FFI), the unraveling of the molecular events underlying this disease has been similarly challenging and unpredictable. In this review we examine the current knowledge of the molecular pathology of FFI and the considerable information that is still missing in order to reach a full understanding of this disease.

Molecular genetics of FFI: a tale of two genotypes

While the first case of FFI that we examined showed selective thalamic atrophy with no changes in the cerebral cortex (15), the second, which had a longer course, also displayed focal cerebral cortical spongiosis consistent with a spongiform encephalopathy, or prion disease (17). This finding prompted the search for a mutation in the prion protein gene (*PRNP*), and led to the discovery of a missense mutation at codon 178, resulting in the replacement of aspartic acid with asparagine in the prion protein (17). At about the same time, Goldfarb et al (12, 19) reported the same mutation in several families that apparently had clinical and pathological features similar to those of Creutzfeldt-Jakob disease (CJD), but quite different from those of FFI. A comparative study of the phenotype in the two diseases showed major differences in both the type and distribution of the histopathological lesions. To gain insight into the molecular basis of the phenotypic heterogeneity associated with the D178N mutation, we carried out allele specific sequencing of the *PRNP* coding region in several subjects affected by either FFI or the CJD-like phenotype, which we named CJD^{178}. We paid special attention to *PRNP* codon 129, the site of a common methionine/valine polymorphism which was previously shown to act as a risk factor in sporadic CJD and to modulate the age of onset in other subtypes of inherited prion diseases (1, 9, 11, 20). In the normal Caucasian population, methionine homozygosi-

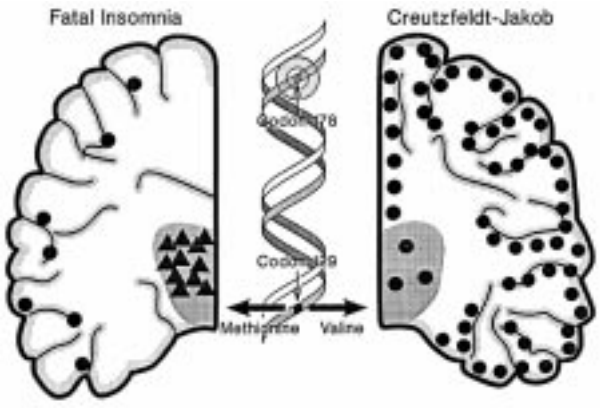

Figure 1. Diagram of the genotype-pathological phenotype correlations in the familial prion diseases linked to the D178N mutation in the PRNP. The codon 129 on the D178N mutated allele determines the phenotype of the disease. Two brain regions (the thalamus, and the cerebral cortex) are represented. In FFI, the thalamus shows moderate to severe atrophy (▲) with no significant spongiform degeneration (●), while the cerebral neocortex has either gliosis or mild to moderate spongiform degeneration depending on the duration of the disease. In contrast, in CJD[178], the cerebral cortex shows prominent spongiform degeneration even in subjects with a relatively short course; spongiform degeneration is also seen in the thalamus. (Ian Warpole ©1993. Reprinted with permission of Discover Magazine.)

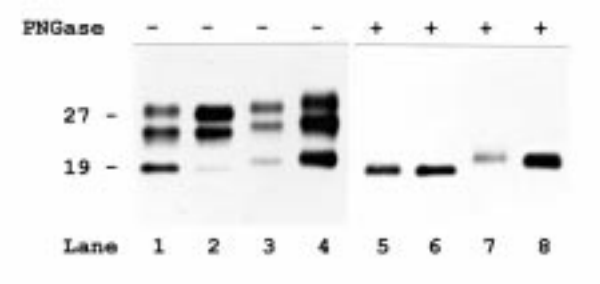

Figure 2. Immunoblot analysis of PrP^{res} extracted from brain of subjects with FFI, CJD[178] or sporadic CJD before (lanes 1-4), and after deglycosylation (lanes 5-8). Lanes 1 and 5: sporadic CJD, V129 V, PrP^{res} type 2; lanes 2 and 6: FFI, M129V, PrP^{res} type 2; lanes 3 and 7 CJD178, V129V, PrP^{res} type 1; lanes 4 and 8: sporadic CJD, M129M, PrP^{res} type 1. Note the comigration of the FFI PrP^{res} and the PrP^{res} type 2 from sporadic CJD, and of the CJD[178] PrP^{res} and the PrP^{res} type 1 from sporadic CJD.

ty at codon 129 is present in 37% of the subjects, methionine/valine heterozygosity in 51% and valine homozygosity in the remaining 12% (20). All FFI subjects showed the methionine codon at position 129 of the mutant allele, whereas all CJD[178] cases had the valine codon (13). Therefore, FFI and CJD[178] segregate with different *PRNP* allelic genotypes or haplotypes, determined by the D178N mutation and the methionine-valine polymorphism at codon 129: the D178N, 129M haplotype segregated with FFI, the D178N, 129V haplotype with CJD[178]. Codon 129 on the normal allele encoded either methionine or valine in both FFI and CJD[178] subjects generating two further subpopulations in each of the two diseases: the methionine homozygotes and the heterozygotes in FFI, and the valine homozygotes and the heterozygotes in CJD[178]. When the two subpopulations were compared, the homozygotes were found to have a shorter clinical course than the heterozygotes in both diseases (13). These findings indicate that codon 129, in conjunction with the D178N mutation, has a twofold role: on the mutant allele it determines the major phenotypic features of the disease, either FFI or CJD[178] (Figure 1), while on the normal allele it modulates the duration, hence the severity, of each of the two diseases. Subsequent studies have confirmed these findings, and, to our knowledge, all subjects with the phenotype of FFI and CJD[178] and the D178N mutation reported to date have been found to carry the D178N, 129M or the D178N,129V haplotype, respectively. Similarly, the mean clinical duration of the disease in the 129 homozygous FFI subjects remains significantly shorter than that of the heterozygotes (see Gambetti and Lugaresi, this issue). Moreover, the modifying effect of codon 129 on the pathological phenotype associated with a pathogenic mutation has now been reported in other inherited prion diseases (14, 34, Parchi et al unpublished).

FFI protein biochemistry: a tale of two proteins

A hallmark of prion diseases is the presence of an isoform of the prion protein that is partially resistant to proteases (PrP^{res}). PrP^{res}, like the normal PrP, usually comprises three major forms, called glycoforms, that are distinguishable by electrophoresis because they carry two, one or no glycans (Figure 2)(5). Since different pathological phenotypes were known to correlate with distinct PrP^{res} in an experimentally transmitted prion disease (2), we postulated that the abnormal prion protein associated with FFI and CJD[178] had different characteristics. Immunoblot analysis of the PrP^{res} fragments extracted from the post-mortem brain demonstrated that the electrophoretic mobility of the PrP^{res}, which is indicative of size, differs in the two diseases (Figure 2) (18). In FFI, PrP^{res} after deglycosylation has a molecular mass of 19 kDa, while in CJD[178] is 21 kDa. In addition, the glycoform ratio of PrP^{res} was distinct in the two diseases (Figure 3) (18). Analysis with endoproteases showed that the two fragments of different size were generated by the cleavage of the PrP^{res} N-terminal at dif-

ferent sites (18). Thus, the different electrophoretic mobility is likely to be the result of distinct conformations of the two PrP^{res}, which expose different cleavage sites to the protease. From these results, it was surmised that the physicochemical properties of PrP^{res} are specified by the D178N mutation in concert with the codon 129 polymorphism, hence, by the primary PrP sequence. More recently, however, two different types of PrP^{res} have also been found in sporadic CJD subjects syngenic in *PRNP* (23). The two PrP^{res} molecules of sporadic CJD, that we named type 1 and 2, differed from each other both in relative electrophoretic mobility and in their glycoform ratio (23). Type 1 and type 2 PrP^{res}, in conjunction with the codon 129 polymorphism, were shown to be associated with distinct clinicopathological variants of sporadic CJD (23). For the first time, in a naturally occurring disorder, it was shown that distinct clinico-pathological variants and PrP^{res} molecules may form independently from the primary structure of PrP (23, 25). Interestingly, type 1 and type 2 PrP^{res} had a molecular mass of either 21 kDa or 19 kDa, indistinguishable from those associated with FFI and CJD^{178} (Figure 2) (23, 24). These observations were followed by the startling finding that the two PrP^{res} types are present in all subtypes of CJD, as well as kuru, independently from the apparent etiology of the disease, i.e. sporadic, inherited or acquired by infection (24, 25).

The discovery of PrP^{res} molecules with distinct physicochemical properties associated with different disease phenotypes has significantly contributed to the understanding of the molecular basis of prion strains. Properties that differentiate the strains, even within an individual host with the same *PRNP* genotype, are the length of incubation time following inoculation, the type and distribution of lesions (neuropathologic profile), and the pattern of intracerebral deposition of PrP^{res} (4) The wide variety of scrapie strains has been traditionally seen as the major challenge to the protein only hypothesis (4). Easily explained by postulating the existence of a nucleic acid as part of the infectious agent, the strain phenomena requires the PrP^{res} to be an informational molecule to fit the prion hypothesis. Can agent strain difference be mediated by variation in PrP^{res} structure rather than by mutations in an agent-specific nucleic acid? Bessen and Marsh identified and characterized two strains of transmissible mink encephalopathy, and showed that when the two strains were propagated in inbred Syrian hamsters, they gave rise to PrP^{res} molecules with distinct electrophoretic mobility and degree of resistance to protease digestion (2). Based on these data, it was proposed that PrP^{res} protein structure determines the molecular basis of strain variation. In this context, the study of FFI and CJD^{178} provided the first identification and characterization of two forms of PrP^{res} in human prion diseases and also showed that in humans pathologically distinct phenotypes may result from different conformers of PrP^{res} (18). The discovery of PrP^{res} type 1 and type 2 associated with distinct clinico-pathological phenotypes in sporadic CJD subjects with the same *PRNP* haplotype provided the first evidence for the existence of prion strains in humans (23).

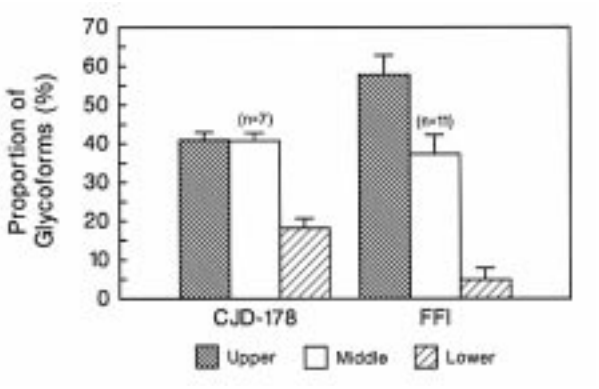

Figure 3. Relative proportion of the three PrP^{res} glycoforms in FFI and CJD^{178}. Mean ± s.d. Upper, high-molecular mass glycoform; middle, low-molecular-mass glycoform; low, unglycosylated form. The PrP^{res} associated with FFI and CJD^{178} have a distinct ratio of glycoforms with underrepresentation of the unglycosylated form.

Experiments of FFI transmission to transgenic mice have given the latest support to the notion that the diversity of prion strains is based on conformation. Brain homogenates from subjects affected by FFI, which contained PrP^{res} type 2, and from subjects with sporadic or familial CJD which contained PrP^{res} type 1 were inoculated to syngenic mice (33). The endogenous PrP^{res} recovered in the affected animals consistently and precisely replicated the size of the corresponding human PrP^{res}. Also, as is typical of different strains, the distribution of lesions in the brain of the injected animal was dependent on the source of the brain homogenate. When mice were injected with FFI brain homogenate, the most significant deposition of PrP^{res} was in the thalamus and rostral region of the corpus callosum. The mice injected with CJD^{200} or sporadic CJD, in contrast, showed widespread distribution of PrP^{res} throughout the brain. By demonstrating the transmission of two distinct disease phenotypes in syngenic animals, these data provided the first demonstration of our original hypothesis that at least two strains of prions affect humans (23, 24). Furthermore, they are consistent with the notion that

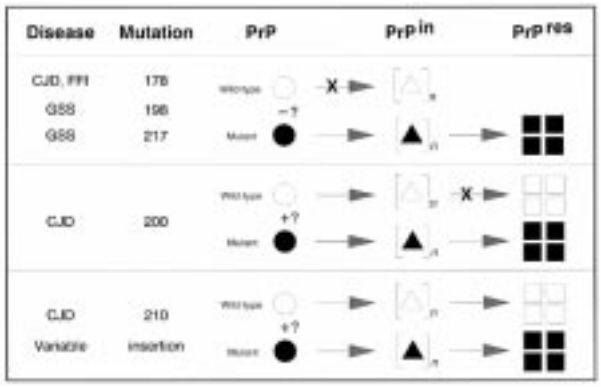

Figure 4. Allelic origin of PrP^{res} in brain of subjects with familial prion diseases. Most subjects with familial prion diseases are heterozygous for the mutation and express PrP derived from both normal and mutant alleles. Mutant PrP is converted to the pathogenic form PrP^{res}, which is both insoluble and protease-resistant. A potential intermediate form, that is detergent-insoluble but not protease-resistant (PrP^{in}), has also been found. After a putative interaction with mutant PrP forms, wild-type PrP expressed by the normal allele may also be converted to PrP^{res} depending on the mutation. In subjects carrying the D178N or E200K mutations PrP^{res} derives exclusively from the mutant allele. In addition, wild-type PrP is found to be insoluble but remains protease-sensitive in subjects carrying the E200K mutation but not in those with the D178N mutation. Similarly, in GSS patients with F198S and Q217R mutations, amyloid PrP peptides (distinct from non-amyloid PrP^{res}) are found to derive from the mutant allele. In contrast, in patients with the V210I or insertional mutation of octapeptide repeats, PrP^{res} derive from both the mutant and the normal allele.

PrP^{res} acts as a template for the conversion of PrP into nascent PrP^{res} during prion replication (33).

Although all such studies support the view that PrP^{res} is the only putative molecule to specify strain variation a cautionary note is needed. Many questions and alternative interpretations of the data remain. For example, it is not yet known whether specific structural properties of PrP^{res} can be attributed to each strain of prions. To reach this goal, the three-dimensional structure of the PrP^{res} associated with the different strains must be established. Alternative explanations for the observed strain-specific PrP^{res} physicochemical properties may be considered. The size of PrP^{res} may well represent a signature imparted by another informational molecule that interacts with PrP. For example, the formation of PrP^{res} type 1 and type 2 may ensue from distinct ligand interactions of PrP.

Allelic origin of PrP^{res} in inherited prion diseases.

An important issue in the understanding of prion diseases and their pathogenesis, particularly for the inherited forms, is to establish the allelic origin of PrP^{res}. FFI and CJD[178], like most of the inherited prion diseases are heterozygous for the pathogenic mutation (26). Thus, the clarification of the allelic origin of PrP^{res} in these patients may shed light to the open question of whether the conversion to PrP^{res} occurs spontaneously based on the mutation itself or requires another, possibly exogenous, factor. We determined the solubility of PrP in detergents, to assess whether PrP is in an aggregated form or not, and whether PrP^{res} derives only from the mutant protein or from both the mutant and normal PrP. We differentiated the mutant from the normal PrP taking advantage of a deletion polymorphism present in the mutant allele of some FFI subjects. The deletion polymorphism in the *PRNP* is known to be present in >1% of the population and has been shown not to alter any of the phenotypic features of the disease (21). In addition, we differentiated the normal and mutant PrP by using an aspartic acid-specific endoprotease, that specifically cleaves the normal but not the mutant PrP (7). We observed that detergent insoluble PrP and PrP^{res} derive exclusively from the mutant PrP in both FFI and CJD[178] regardless of whether the affected subjects are homozygous or heterozygous at codon 129 (Figure 4)(7).

Variable findings concerning the participation of the normal PrP in PrPres formation have been obtained in other inherited prion diseases (Figure 4). For example, in the CJD subtype linked to the E200K mutation only the mutant PrP is resistant to proteases, but both mutant and normal PrP are insoluble in nonionic detergents, indicating that wild type PrP, although not converted to PrPres, is in an aggregated abnormal form likely to be pathogenic (10). In the CJD subtype associated with the V210I mutation, and in the inherited prion diseases linked to insertional mutations of five and six extra octapeptide repeats, both wild type and mutant PrP are converted (7, 29, 30). Finally, in two subtypes of Gerstmann-Sträussler-Scheinker disease (GSS) the amyloid deposits that characterize these diseases contain exclusively the mutant PrP (31). Thus, in inherited prion diseases, the contribution to PrPres by the normal allele is mutation specific. The apparent lack of modification of the wild type PrP in FFI and CJD[178] might be unique or unusual among nonamyloidogenic mutations. Whether the low amount of PrPres in FFI is due to monoallelic origin of PrPres or to other mechanisms remains to be determined. The transmissibility of diseases such as FFI and CJD[178] in which only PrPres that derives from mutant PrP is found, is puzzling. It is, in fact, unclear why the normal PrP is converted after transmission, while this is not the case in FFI patients. This apparent discrepancy might be due to the PrPres

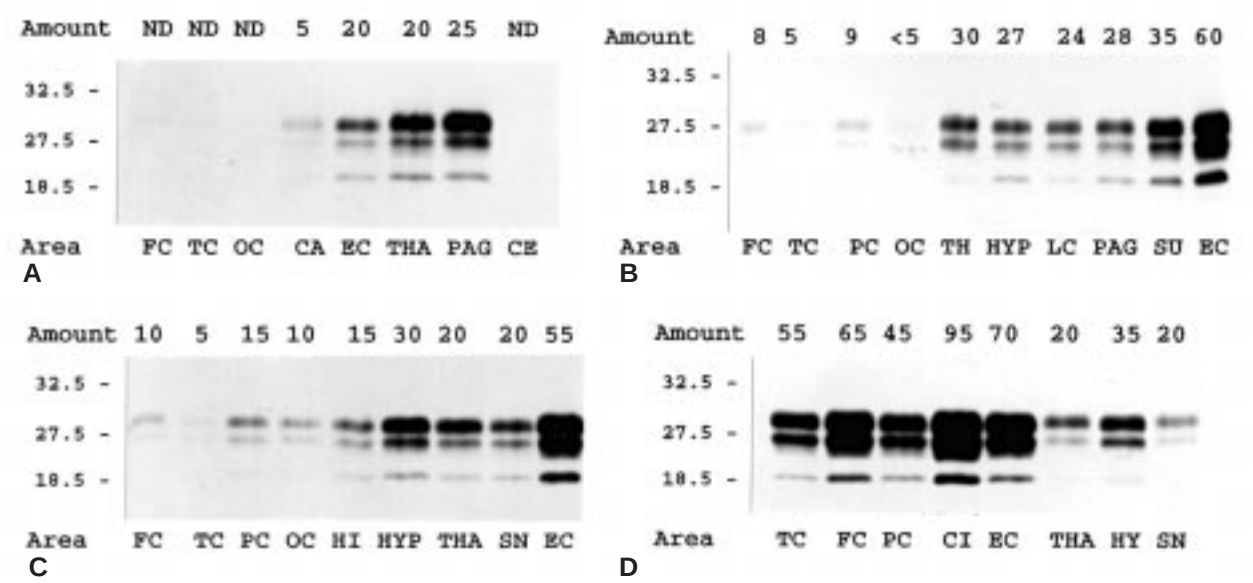

Figure 5. Quantitative immunoblot analysis of PrP^{res} extracted from different brain regions of 4 FFI affected subjects with different duration of symptoms and/or codon 129 genotype in the normal *PRNP* allele. Molecular mass standards are indicated at the left, and the amount of PrP^{res}, calculated by densitometric analysis, are indicated on the top of each immunoblot. Brain regions are abbreviated as follows: frontal cortex (FC), temporal cortex (TC), parietal cortex (PC), occipital cortex (OC), entorhinal cortex (EC),CA1 region of the hippocampus (HI), subiculum (SU) caudate nucleus (CA), mediodorsal thalamic nucleus (THA or TH), hypothalamus (HY or HYP), midbrain periacqueductal gray (PAG), substantia nigra (SN), locus coeruleus (LC), cerebellum (CE). (A) Patient with a duration of symptoms of 7 months, homozygous for methionine at codon 129. (B) Patient with a duration of symptoms of 11 months, homozygous for methionine at codon 129. (C) Patient with a duration of symptoms of 12 months, heterozygous at codon 129. (D) Patient with a duration of symptoms of 25 months, heterozygous at codon 129.

administration by intracerebral injection which results in the local accumulation of a relatively large amount of PrPres. The high concentration of mutant PrPres might overcome in the experimental animal the normal PrP barrier present in FFI. Additional studies are needed to clarify this issue.

Histopathology and distribution of the protease-resistant PrP

Although severity and topography of the lesions may vary as a function of the disease duration, which, in turn, is related to the methionine/valine polymorphism at codon 129, the pathologic phenotype of FFI shows highly characteristic and consistent features. The thalamus displays loss of neurons and astrogliosis in all subjects regardless of the duration (16, 22). The medio-dorsal and anterior ventral thalamic nuclei are invariably affected, perhaps more severely in the homozygotes, while the involvement of other thalamic nuclei varies. The inferior olives also show neuronal loss and gliosis in all cases. In contrast, the pathology of the cerebral cortex varies in proportion to the disease duration and is more severe in the limbic lobe than in the neocortex (16, 22). The entorhinal cortex shows spongiosis and

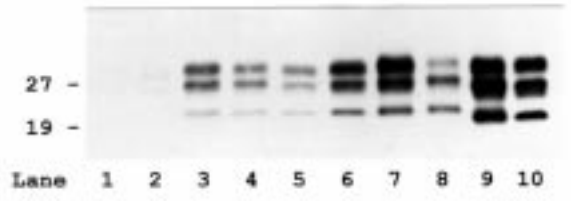

Figure 6. Quantitative immunoblot analysis of PrP^{res} in FFI and CJD. Two samples containing the highest amount of PrP^{res} detected to date by us in FFI subjects homozygous (lane 1) or heterozygous (lane 2) at codon 129 respectively, are compared with samples of cerebral cortex from subjects with CJD^{178}, CJD^{200} and sporadic CJD. Each lane has been loaded with the same amount of brain homogenate (equivalent to 0.2 mg of wet tissue). The amount of PrP^{res} is significantly higher in sporadic or familial CJD than in FFI. Lane 1: FFI, M129M; lane 2: FFI, M129V; lanes 3-5: CJD^{178}, V129V and V129M; lanes 6,7: CJD^{200}, M129M, PrP^{res} type 1; lane 8: sporadic CJD, M129M, PrP^{res} type 1, lane 9: sporadic CJD, V129 V, PrP^{res} type 2; lane 10: sporadic CJD, M129M, PrP^{res} type 2.

astrogliosis in virtually all subjects. In contrast, the neocortex is essentially spared in the subjects with a disease duration of less than one year, which are, in the large majority, homozygous for methionine at codon 129;

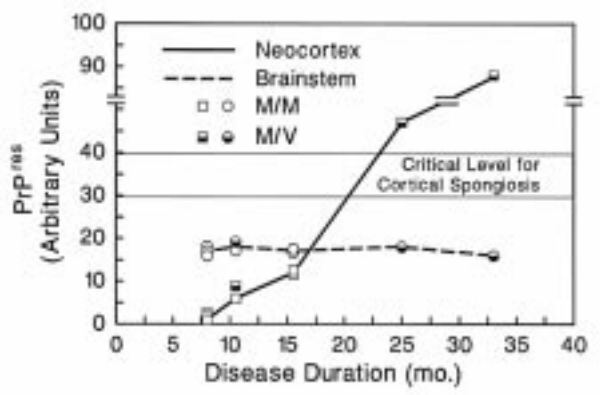

Figure 7. Different timing and rate of PrP^{res} deposition in distinct brain regions of 9 FFI subjects. In the cerebral neocortex, PrP^{res} is detectable in significant amount only in patients with a duration of symptoms longer than 8 months and increases with disease duration. In contrast, the brainstem shows similar amounts of PrP^{res} regardless of the duration of symptoms. M/M: homozygotes for methionine at codon 129; M/V: heterozygotes at codon 129.

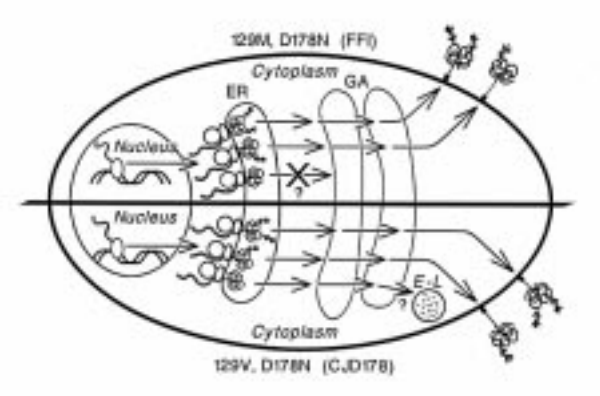

Figure 8. Processing of mutant PrP in a FFI and CJD^{178} cell model. PrP undergoes post-translational modifications including addition of the glycosylphosphotidyl inositol (GPI) anchor and of the high mannose glycan cores in the endoplasmic reticulum (ER). The glycans are then modified to complex sugar moieties in the Golgi Apparatus (GA) and PrP is transported to the cell surface. The unglycosylated form of both D178N mutants is underrepresented at the cell surface. A fraction of the unglycosylated D178N FFI protein might be degraded in the ER, while the D178N CJD protein is apparently degraded in a more distal location, possibly the endosomal-lysosomal (E-L) compartment.

however, the neocortex is focally affected by spongiosis and gliosis in subjects with a course between 12 and 20 months, which include homozygous and heterozygous subjects, and diffusely involved only in subjects with a disease of more than 20 months duration, which are almost exclusively homozygous (22). In addition, the frontal, temporal, and parietal lobes are affected more severely than the occipital lobe. Finally other structures, such as the midbrain periacqueductal gray, the superior colliculus, and the hypothalamus show focal pathology, usually characterized by astrogliosis (22). Overall, the thalamus is more severely and consistently involved than any other brain region. Therefore, on the basis of the pathology, FFI can be defined as a preferential thalamic degeneration.

The study of the regional distribution of PrP^{res} has also provided provocative data (Figures 5 and 7) (22). PrP^{res} deposition in FFI is more widespread than that of the histologic lesions and heavily influenced by the duration of the disease, particularly in the cerebral cortex. In the subjects with a 7-8 month duration, PrP^{res} is detected in significant amounts only in limbic areas such as the entorhinal cortex or the cingulate gyrus, and in subcortical structures including the thalamus, the hypothalamus and the brainstem (Figure 5A). As duration increases, however, the abnormal protein becomes progressively more detectable in the cerebral neocortex, and eventually accumulates in significantly higher amounts in the cerebral cortex than in subcortical regions (Figure 5). The apparent kinetics of accumulation of the abnormal protein varies among different brain regions (Figure 7)(22). While in the neocortex and, to a lesser extent, in the limbic cortex and the striatum, the amount increases with the duration of symptoms, in some subcortical regions, such as the brainstem and the thalamus, it is found in similar amounts in all subjects, regardless of the duration of the disease. Thus, it appears that, despite the ubiquitous presence of the D178N mutation, PrP^{res} deposition selectively starts in subcortical regions and only later spreads to cerebral cortex. The study of the distribution PrP^{res} has also provided an explanation for the absence or mildness of spongiform degeneration that characterize the FFI phenotype (22). We have shown that in both FFI and sporadic CJD there is a good correlation between the amount of PrP^{res} and the presence and severity of spongiform degeneration (Figure 7) (22, 23). By comparing samples from FFI and other subtypes of human prion diseases, we (22) and others (3) have found that the overall amount of PrP^{res} present in FFI is, on average, 5 to 10 times less than that detected in the most common subtypes of CJD (Figure 6). Thus, the overall rate of accumulation of the abnormal protein, in addition to PrP^{res} physicochemical properties and its regional distribution, specifies the phenotypic variability of human prion diseases.

Cell model of FFI and CJD[178]

In order to investigate the early events and the predisposing factors leading to the conversion of mutated PrP in FFI and CJD[178], we developed human neuroblastoma cell lines that expressed either the homologous haplotype associated with FFI (D178N, 129M) or the haplotype associated with CJD[178] (D178N, 129V) (27). PrP^{C} is normally synthesized in the endoplasmic reticulum, modified in the Golgi apparatus, and transported to the cell surface where it is bound by a glycophosphatidyl inositol (GPI) anchor (Figure 8). Using immunoblotting, and a pulse-chase paradigm, we showed that, compared to the wild type PrP, only one third of the mutant PrP(PrPM) reaches the cell surface. In addition, we found that the unglycosylated form is preferentially underrepresented in both diseases models, although more severely in FFI (27). The pulse- chase experiment, which follow synthesis, maturation and transport of the PrP molecules showed that the synthesis and early metabolism of the the three mutant glycoforms is not different from those of the wild type. After 2 hours chase, however, when only mature forms of PrP are detected, the unglycosylated and, to a lesser extent the intermediate PrPM forms, decrease significantly. In contrast to the pulse-chase experiment, immunoblotting, which provides information on the proteins at steady state, showed that all three mutant PrP glycoforms are present and well represented in the intracellular compartment (Petersen et al unpublished data).The relative decrease in the unglycosylated and intermediate forms during the pulse-chase experiments, which depend on immunoprecipitation, and their appearance in the immunoblot suggested that these two glycoforms might be, at least in part, aggregated and therefore are not immunoprecipitable or transportable to the cell surface. To assess the aggregation state of the mutant PrP we performed detergent extraction experiments (6, 28, 32). We observed that in the mutant cells, the unglycosylated and intermediate forms were more prone to aggregation than the highly glycosylated and the corresponding glycoforms of the wild type cells. Moreover, we found that a minute amount of mutant PrP was resistant to a ten minute digestion with 3.3 μg/ml of proteinase K. The relevance of this finding to the PrP^{res} present in FFI brains is questionable since for digestion of brain samples proteinase K is used at 50-100μg/ml or more for one hour at 37°C. The weakly protease resistant mutant PrP may simply reflect aggregation and is unlikely to be an early form of the disease-associated PrP^{res}.

To validate our cell model, we examined the glycoform ratio of the protease-sensitive mutant PrP (not PrP^{res}) in FFI brain regions essentially devoid of PrP^{res}. The unglycosylated form of the mutant PrP was also preferentially underrepresented in the membrane fraction isolated from FFI brains but not from brains of control subjects (27). This finding indicates that the underrepresentation of the unglycosylated form of PrP^{res} in FFI and CJD[178] is due to the relative unavailability of this form for conversion to PrP^{res} as a consequence of the mutation, rather than the preferential conversion of the highly glycosylated forms to PrP^{res}. Moreover, since the unglycosylated isoform of the mutant PrP is markedly underrepresented at the cell surface in the FFI and CJD[178] cell models and in the membranes of the FFI brains, it is tempting to speculate that the conversion of the mutant PrP to PrP^{res} takes place at the plasma membrane.

Unsolved issues in FFI

Major issues remain to be solved in order to reach a clear understanding of the pathogenesis of FFI. The first question concerns the potential effects of the mutant PrP on protein processing, cell structure and function. Our cell model indicates that in FFI the D178N mutation, probably through an effect on conformation, destabilizes the mutant PrP. Consequently, only 30% of the mutant PrP reaches the cell surface, allegedly the site of normal PrP function, while the remaining aggregates or is degraded, probably by the 'quality control' system of the cell, a mechanism thought to promote the repair or breakdown of harmful proteins in the endoplasmic reticulum (27). In addition, the protein that reaches the cell membrane shows a significant alteration in the ratio of glycoforms. The effect of these changes, however, remain difficult to assess at molecular level because the function of the normal PrP is not known. Sophisticated clinical tests, such as polysomnography and positron emission tomography (PET), have failed to demonstrate any abnormality in carriers of the FFI before the clinical onset of the disease, suggesting that the dysmetabolism of the mutant PrP has no major dysfunctional effects. Since in patients with clinical symptoms, even in cases of very short duration, PrP^{res} is invariably detectable, it may be surmised that the clinical onset of the disease only occurs when a sufficient amount of PrP^{res} is formed. How the conversion of the mutant PrP to PrP^{res} occurs and why it occurs only at a relatively advanced age, remains to be determined.

A second major unsolved issue in the pathogenesis of FFI is how the two haplotypes D178N, 129M of FFI and D178N, 129V of CJD[178], that differ only in the presence of a nonpathogenic codon 129 can specify two PrP^{res} that

conceivably have different conformations and cause two diseases with distinct clinico-pathological phenotypes. In the original study reporting the two haplotypes, we postulated that asparagine, present at position 178 of the mutant PrP in both diseases, interacts differently with the methionine and valine residues present at position 129 in FFI and CJD[178], respectively, and resulted in two distinct abnormal PrP conformers, which, in turn, caused distinct diseases (13). Recently, in a nuclear magnetic resonance (NMR) study of the structure of the mouse recombinant PrP fragment from residue 121 to residue 231, Riek et al. observed that although there is no direct contact, the side chains of residues 129 and 178 are connected by a hydrogen bond which could mediate interactions between these two residues (29). Although these studies were carried out with wild type PrP, they are consistent with the original hypothesis that interactions between residues 129 and 178 are promoted by the D178N mutation and are different according to the residue, methionine or valine, present at position 129. Alternative mechanisms, however, should be also considered. First among these is the possibility that the codon 129 polymorphism, alone in sporadic CJD or in conjunction with mutations in the familial forms, modulates the interaction with another molecule that specifies, at least in part, the PrP^{res} physicochemical properties and the disease phenotype. This hypothesis seems to be supported by the most recent studies of the molecular pathology of human prion diseases, which show that distinct clinico-pathological variants and PrP^{res} molecules may form independently from the primary structure of PrP, and that the codon 129 plays a role as a modulator of disease phenotype not only in association with the D178N mutation, but also in several other human prion diseases (14, 23, 25, 36, Parchi et al. unpublished).

The mechanisms regulating the topography of PrP^{res} formation and its relationship with neuronal dysfunction, histopathology and ultimately the clinical course of the disease in FFI remain enigmatic. As mentioned before, in FFI the distribution of PrP^{res} as well as that of the hypometabolic brain areas detected by PET scan are more widespread than the histopathological lesions (8, 22). In addition, there is an incomplete correlation between levels of PrP^{res} and severity of histopathology. For example, the thalamus and the brain stem contain similar amounts of PrP^{res}, although the thalamic lesions are much more severe. Similarly, apoptosis, a recent finding in FFI (see Dorandeu et al, this issue), does not correlate well with the amount of PrP^{res}. Although these observations suggest that PrP^{res} accumulation precedes the histopathological lesions and is the cause of neuronal dysfunction, they also indicate that there is a selective vulnerability to PrP^{res} or other factors among distinct brain regions that needs to be investigated.

The different duration of symptoms and clinical phenotypes among subjects homozygous and heterozygous at codon 129 is also puzzling. There is no overall correlation with the severity of the pathology or the degree of PrP^{res} accumulation, since the subjects with the shortest course (mostly homozygotes) have the least amount of PrP^{res} and pathology in most brain regions (22). In the thalamus or the brain stem, however, similar amounts of PrP^{res} are found in all subjects, regardless of the disease duration. Thus, one explanation is that in these regions the kinetics of PrP^{res} formation is different between homozygotes and heterozygotes and that this affects in some way the clinical course of the disease. Alternatively, PrP^{res} accumulation or other factors yet unidentified, impair the neuronal function before the appearance of significant pathology and this effect is more pronounced in the homozygotes than in the heterozygotes.

These are some of several issues that need to be investigated in order to gain a clear understanding of the molecular mechanisms involved in the pathogenesis of FFI. Their clarification will further deepen our knowledge of this fascinating disease.

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